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ORIGINAL CONTRIBUTION

Pharmacokinetics and bioavailability of hydroxytyrosol are dependent on the food matrix in humans

Carolina Alemán-Jiménez[1] · Raúl Domínguez-Perles[2] · Sonia Medina[2] · Iva Prgomet[3] · Iván López-González[2] · Agustín Simonelli-Muñoz[1] · Maria Campillo-Cano[1] · David Auñón[4] · Federico Ferreres[2] · Ángel Gil-Izquierdo[2]

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Abstract

Purpose Several studies have demonstrated the properties of hydroxytyrosol, a phenolic compound present in olive oils and olives with a well-characterized impact on human health. Nevertheless, some knowledge gaps remain on its bioavailability and metabolism; overall concerning to the real rate per cent of absorption and biovailability of dietary hydroxytyrosol and the influence of the dietary food-containing hydroxytyrosol on it.

Methods A double-blind study was performed including 20 volunteers who ingested 5 mg of hydroxytyrosol through diverse food matrices, to discover the influence on pharmacokinetics and bioavailability of HT metabolites (hydroxytyrosol acetate, 3,4-dihydroxyphenylacetic acid (DOPAC), tyrosol, and homovanillic alcohol) of the distinct matrices by UHPLC–ESI–QqQ–MS/MS.

Results The HT pharmacokinetics after consumption of different food matrices was strongly dependent on the food matrix. In this aspect, the intake of extra virgin olive exhibited significantly higher plasma concentrations after 30 min of oral intake (3.79 ng/mL) relative to the control. Regarding the hydroxytyrosol bioavailability, the intake of extra virgin olive oil, as well as fortified refined olive, flax, and grapeseed oils provided significantly higher urinary contents (0.86, 0.63, 0.55, and 0.33 µg/mg creatinine, respectively) compared with basal urine, whereas hydroxytyrosol metabolites showed no significant changes. No differences were found between men and women.

Conclusions The metabolic profile of hydroxytyrosol is influenced by the food matrix in which is incorporated, with the oily nature for the final bioavailability being relevant. Extra virgin olive oil was identified as the best matrix for this compound. The results described contribute to the understanding of the relevance of the food matrices for the final absorption of hydroxytyrosol and hence, the achievement of the highest health protection potential.

Keywords Hydroxytyrosol metabolites · Human · In vivo · Food matrix · Bioavailability · Pharmacokinetic

Abbreviations

Amu	Atomic mass unit
DOPAC	3,4-Dihydroxyphenylacetic acid
HT	Hydroxytyrosol

Carolina Alemán-Jiménez and Raúl Domínguez-Perles contributed equally to the present work.

✉ Sonia Medina
smescudero@cebas.csic.es

✉ Ángel Gil-Izquierdo
angelgil@cebas.csic.es

[1] E.U. Human and Dietetic Nutrition, San Antonio Catholic University, Campus Los Jerónimos, s/n, 30107 Murcia, Spain

[2] Research Group on Quality, Safety, and Bioactivity of Plant Foods, Department of Food Science and Technology, CEBAS-CSIC, Edif. 25, 30100 Espinardo, Murcia, Spain

[3] Centre for the Research and Technology of Agro-Environmental and Biological Sciences, CITAB, University of Trás-Os-Montes e Alto Douro, UTAD, Quinta de Prados, 5000-801 Vila Real, Portugal

[4] Department of Food Technology and Nutrition, Molecular Recognition and Encapsulation Group (REM), San Antonio Catholic University, Campus Los Jerónimos, s/n, 30107 Murcia, Spain

HTA	Hydroxytyrosol acetate
HValc	Homovanillic alcohol
MRM	Multiple reaction monitoring
SPE	Solid phase extraction
Tyr	Tyrosol
UHPLC–ESI–QqQ–MS/MS	Ultra-high performance liquid chromatography coupled to electrospray ionization and triple quadrupole mass spectrometry

Introduction

Traditionally, the health benefits attributed to the Mediterranean diet have been associated with a reduction in the consumption of saturated fats and their replacement with an increased intake of olive oils, which feature high concentrations of bioactive compounds [1]. In this respect, one of the most deeply characterized phytochemicals in these plant-foods is hydroxytyrosol (HT), a phenolic compound that is naturally present in olive oils and table olives. Its importance as a healthy replacement is supported by a number of reports demonstrating the properties of HT as a powerful antioxidant, with broad positive impacts on human health [2, 3]. Based on these experimental characterizations, the interest on this compound has grown in the last few decades, as it also features outstanding anti-inflammatory, antimicrobial, and neuroprotective activities, which confer HT with a central role in the prevention of cardiovascular and neurodegenerative diseases, and metabolic syndrome as well [4–6]. These attributes have prompted the European Food Safety Authority to publish positive scientific opinions highlighting the interest of dietary HT to protect low density lipoproteins (LDL) and thus, to reduce the incidence of cardiovascular pathologies, pointing out that the dietary consumption of 5 mg is enough to achieve these benefits [7].

To understand the biological activity of HT, some light must be shed on its metabolism and distribution over diverse tissues, as well as on its capacity to cross the blood–brain barrier [8]. In this regard, the bioavailability and bioactivity of phenolic compounds depend on a myriad of factors associated to human physiology, such as their stability under gastrointestinal conditions, the efficiency of intestinal absorption, the composition of the intestinal microbiota, and the post-absorption metabolism, among other issues [9]. At present, it has been demonstrated that after dietary ingestion, phenolic compounds are metabolized in two phases: the first phase is the hydrolysis that occurs in the stomach and small intestine, and where most HT is rapidly absorbed; and the second phase, a conjugation reaction that occurs in the intestinal epithelium and hepatocytes [10, 11]. Moreover, additional factors, such as the physico-chemical characteristics of the food matrix in which bioactive compounds are present, seem to critically influence its bioavailability, although these factors have not yet been fully explored as of today [12, 13], and less so at dietary concentrations.

In addition, sex has been proposed as a critical factor which conditions the final availability of HT, which seems to be associated to a differential metabolism and enterohepatic circulation which in turn has a direct impact on the bioavailability of these compounds. These differences have been associated to higher urine concentrations in female plasma and urine, which was demonstrated upon an in vivo assay in a murine model [10]. Nonetheless, to date, no references of this are available regarding humans.

To provide a new perspective for understanding the mechanism through which bioactive compounds of olive oil act in vivo, the present article uncovers the ratio of absorption of HT and its conversion into its main metabolites, hydroxytyrosol acetate (HTA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic alcohol (HValc), and tyrosol (Tyr), in humans, throughout the 24 h following the ingestion of 5 mg of HT through diverse oily and aqueous matrices (extra virgin olive oil, refined olive oil, flax oil, grapeseed oil, margarine, and pineapple juice). Hence, metabolites were analysed in plasma and urine using the robust and sensitive method of Ultra-high performance liquid chromatography coupled to a Mass Spectrometer equipped with an electrospray ionisation (ESI) chamber and triple quadrupole mass analyser for tandem analysis (UHPLC-ESI-QqQ-MS/MS) [10].

Material and methods

Reagents

Hydroxytyrosol (HT), hydroxytyrosol acetate (HTA), and 3,4-dihydroxyphenylacetic acid (DOPAC) were provided by SEPROX Biotech S.L. Tyrosol (Tyr) and homovanillic alcohol (HValc) were purchased from Sigma-Aldrich (St. Louis, MO, USA), as well as the hydrolytic β-glucuronidase, type H2 from *Helix pomatia* and Bis–Tris (bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane). All LC–MS grade solvents were obtained from J.T. Baker (Phillipsburg, New Jersey, USA). Formic acid and hydrochloric acid were purchased from Panreac (Castellar del Vallés, Barcelona, Spain). The solid phase extraction (SPE) cartridges used in this study (Strata X-AW, 100 mg/3 mL) were obtained from Phenomenex (Torrance, California, USA).

Study design

We hypothesized that the food matrix has a direct influence on the intestinal absorption of HT and its metabolites, and

thus on the plasma and urine concentration of these compounds. To test this initial hypothesis, the design was outlined as a double-blind study in which 20 human volunteers ingested 5 mg of hydroxytyrosol through a single dose of diverse food matrices (extra virgin olive oil, and five fortified food matrices (refined olive oil, flax oil, grapeseed oil, margarine, and pineapple juice)). After each intervention, all the volunteers underwent a wash-out period of 96 h to avoid interferences among the different matrices assayed. Pharmacokinetic and bioavailability results were compared with the baseline control (BC) values, recorded from samples collected before ingesting each matrix, and an additional intervention day, when volunteers ingested non-fortified refined olive oil. Peripheral blood samples were collected at time-points (min) 0 (BC), 30, 60, 120, 180, and 240. Urine samples were collected at rest (basal urine) and under fasting conditions (24-h urine) (Fig. 1). The study was carried out on 20 Caucasian volunteers (10 men and 10 women), aged 19–23 years with normal BMI > 18.5 and < 25 kg/m^2 from the San Antonio Catholic University of Murcia (Spain), who agreed to participate in the project. All subjects fulfilled the following eligibility criteria: non-smokers, follow stable food habits, and did not receive any medication during the experimental procedure. The study was approved by the Bioethics Committee of the Catholic University "San Antonio" of Murcia in accordance with the Declaration of Helsinki., and all volunteers signed written informed consent forms. The dietary habits of the subjects were monitored during the entire assay, including wash-out and baseline periods, to avoid the ingestion of polyphenol-containing foods such as tea, chocolate, coffee, fruits or juices, vegetables, soya, berries, EVOO (extra virgin olive oil) and alcoholic drinks (including wine and beer) during the 3 days previous to each intervention, in agreement with previous reports including nutritional intervention studies with human volunteers who consumed HT-based foods that could interfere with the results obtained [14, 15].

Sample collection and preparation

A range of clinical analyses (including haematology, biochemistry, and urine chemical analysis) were performed to monitor the health status of the volunteers. Peripheral blood samples were collected at the University Hospital

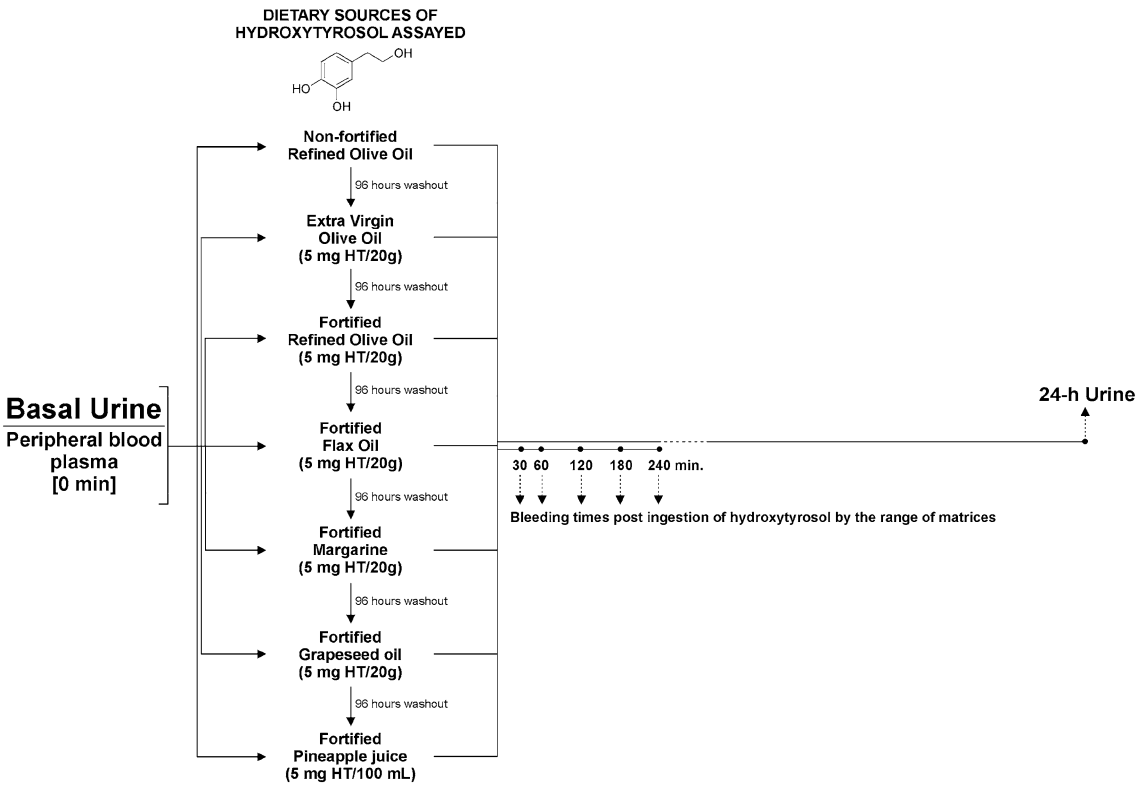

Fig. 1 Study design. This was a double-blind study. Twenty volunteers (10 men and 10 women) ingested 5 mg of hydroxytyrosol added to 6 diverse food matrices (extra virgin olive oil, refined olive oil, flax oil, grapeseed oil, margarine, and pineapple juice). After each intervention, all volunteers underwent a wash-out period of 96 h to avoid interferences among the different matrices assayed. Two controls were included in the experimental design, baseline control (BC) and non-fortified refined olive oil. Peripheral blood samples were collected at time-point (min) 0 (BC), 30, 60, 120, 180, and 240. Urine samples were collected at rest (basal urine) and under fasting conditions (24-h urine)

under fasting conditions at the beginning of each day of intervention. Hence, blood samples at rest and at the different time-points described in the experimental design (Fig. 1) were obtained by venipuncture and placed in different tubes according to the analytical procedures foreseen. For the biochemical analysis, glucose (mg/dL), urea (mg/dL), total cholesterol (mg/dL) and HDL- and LDL-cholesterol (mg/dL), as well as haematological analysis were carried out using a Cobas 8000 c702 module (Roche Diagnostic, Mannheim, Germany). Immediately upon collection, for HT and HT derivatives analysis, blood from citrated blood vacutainers was centrifuged at 2000×*g* for 10 min at 4 °C to obtain plasma and then transferred to polypropylene tubes with 10% L-ascorbic acid and 0.58 M acetic acid as preservatives and stored at − 80 °C until final processing and analysis.

Twenty-four-hour urine samples were collected before and after each intervention day in sterile, dark polystyrene, tubes (2 L) with screw caps with 10% L-ascorbic acid as the chemical preservative. The urine analysis was also performed in a Cobas 8000 c702 modular analyser (Roche Diagnostic, Mannheim, Germany) to test the content of creatinine (mg/dL) and albumin (mg/L). The concentration of HT and HT derivatives analysed in the 24-h urine was normalised as micrograms per milligram of creatinine (µg/mg creatinine).

For the analysis of the concentration of HT, HTA, DOPAC, Tyr, and HValc, plasma and urine samples were thawed at room temperature and centrifuged (8538 ×*g* for 5 min). Samples (100 and 400 µL, respectively) were processed following the methodology previously described by Dominguez-Perles et al. [10]. Briefly, the supernatants were hydrolysed by incubation with 300 UI (plasma) and 1500 UI (urine) of β-glucuronidase from *Helix pomatia* for 2 h at 37 °C, clarified with 200 µL of MeOH/HCl (200 mM) and centrifuged at 8538× *g* for 5 min. The supernatants were cleaned up by SPE, using Strata X-AW cartridges, which were conditioned and equilibrated with 2 mL of MeOH/formic acid (98:2, *v/v*) and 2 mL of water/ formic acid (98:2, *v/v*), respectively. Then, the samples were loaded and the SPE cartridges were washed with water/formic acid (98:2, *v/v*). The target analytes were eluted with 1 mL of MeOH/formic acid (98:2, *v/v*) and dried with a SpeedVac concentrator (Savant SPD121P; Thermo Scientific, Waltham, MA). After that, the extracts were reconstituted with 200 µL of solvent A/B (90:10, *v/v*) used as the mobile phases for the UHPLC/MS/MS analyses. Quality control (QC) samples were prepared with HT and HT derivatives spiked in baseline control (BC) plasma and urine samples at two different concentrations (1 ng/mL (corresponds to the limit of quantification (LOQ)) and 5 ng/mL). Both precision (CV < 15%) and accuracy (80–120%) parameters in three replicates were acceptable according to ICH and FDA guidelines (data not shown).

UHPLC–ESI–QqQ–MS/MS analysis

The plasma and urinary metabolites (HT, HTA, DOPAC, Tyr, and HValc) were analysed using the previously cited methodology [10]. In brief, plasma and urinary HT and HT derivatives were analysed using an UHPLC–MS/MS with triple quadrupole technology (Agilent Technologies, Waldbronn, Germany). The chromatographic separation was carried out on the analytical column BEH C18 1.7 µm (2.1 × 50 mm) (Waters, Milford, M.A.) using water/formic acid (99.9:0.1, *v/v*) (A) and MeOH (B) as mobile phases. The flow rate was 0.4 mL/min, using a linear gradient (t; %B): (0; 20), (6; 95), (7; 100), (10; 20), and the injection volume was 10 µL. For the qualitative analyses, the target analytes were identified according to the most-abundant product ions detected by multiple reaction monitoring (MRM) in positive and negative modes depending on the analyte considered (Table 1). To avoid that the use of β-glucuronidase (with sufatase activity) from *Helix pomatia* may result in an underestimation of the bioavailability of phenolic compounds upon oral administration due to incomplete hydrolysis of conjugate compounds, previous assays with different times of hydrolysis and enzymatic units of this enzyme have been carried out and have already been used in other bioavailability studies of hydroxytyrosol performed at our laboratory [10, 16] (In this sense, the current study also monitored the potential residual conjugate compounds by theoretical MRM transitions because of the lack of commercially-available standards). Thus, $[M–H]^-$ 176 atomic mass units (amu) (glucuronide), 352 amu (diglucuronide), 80 amu (sulfate), 160 amu (disulfate), and 256 amu (sulfoglucuronide) were checked. The quantitation of HT, HTA, DOPAC, Tyr, and HValc was achieved by comparison with standard curves which were freshly prepared each day of analysis using authentic standards.

Statistics

All assays for each volunteer ($n = 20$) were performed in triplicate and concentrations were provided as means ± standard

Table 1 UHPLC-ESI-QqQ-MS/MS parameters for the quantification of hydroxytyrosol (HT), hydroxytyrosol acetate (HTA), 3,4-dihydroxyphenylacetic acid (DOPAC), tyrosol (Tyr), and homovanillic alcohol (HValc)

Analyte	Retention time [min]	ESI mode	MRM transition
DOPAC	0.535	Positive	169 > 123
HTA	0.542	Positive	195 > 91
HT	1.214	Negative	153 > 123
HValc	1.603	Positive	169 > 151
Tyr	1.848	Positive	139 > 121

deviation (SD). Differences concerning plasma and urine concentration of HT derivatives were analysed with unpaired *t*-Student tests and analyses of variance (ANOVA). The fulfilment of the one-way ANOVA requirements, specifically the normal distribution of the residuals and the homogeneity of variance, was tested with the Kolmogorov–Smirnov (with Lilliefors correction) and Levene's tests, respectively. When statistical differences were identified, the variables were compared using Tukey's multiple range test by utilizing the separate food matrices as the sources of variation. The analyses were carried out with IBM SPSS statistics 24.0 (SPSS Inc., Chicago, IL, USA). Significant differences among means were considered at $p < 0.05$.

Results

The qualitative analysis of the biological samples (plasma and urine) collected for detecting the presence of HT and HT metabolites (HTA, DOPAC, HValc, and Tyr) was carried out by an ultra-high performance chromatography coupled to electrospray ionization and mass spectrometry with triple quadrupole technology (UHPLC-ESI-QqQ-MS/MS). Their assessment was performed after enzymatic hydrolysis of plasma and urine, which allowed monitoring free HT, HTA, DOPAC, HValc, and Tyr, after the intake of the different matrices considered. Furthermore, conjugate compounds after enzymatic hydrolysis were not present and therefore, no incomplete hydrolysis by β-glucuronidase was observed.

The retention times matched for both authentic standards and compounds present in the biological samples (0.535, 0.542, 1.214, 1.603, and 1.848 min for DOPAC, HTA, HT, HValc, and Tyr, respectively) (Table 1). For this, the parent mass and fragmentation pattern for HT metabolites were recorded by applying positive or negative ionization modes, depending on the metabolite monitored, in agreement with the method described by Dominguez-Perles et al. (2017) [10], which allowed their accurate quantification (Table 1).

Plasma kinetics of hydroxytyrosol after its oral administration through diverse food matrices

The UHPLC–ESI–QqQ–MS/MS-based assessment of peripheral blood plasma on the concentration of HT was performed at 0 (BC), 30, 60, 120, 180, and 240 min post-intake of 5 mg of HT in the array of matrices considered, corresponding to the equivalent dose recognized to promote human health according to EFSA [7]. To contrast the pharmacokinetic effect of the oily matrices, non-supplemented refined olive oil was also administered to the volunteers.

The plasma concentration of HT (Fig. 2) was achieved after enzymatic hydrolysis, which allowed registering the total amount of the target analyte. This approach provided plasma concentrations that were higher to the quantification limit of the method utilized (1 ng/mL) [10], which was the same as another methodology published for the analysis of free HT in human plasma following the administration of olive oil [17]. The highest plasma concentration of HT was achieved at 30 min after its oral intake, in agreement with previous studies found in the literature reviewed [15, 18]. Hydroxytyrosol was rapidly removed from the plasma, showing no significant differences with the plasma concentration values found in the negative control (non-supplemented refined olive oil) after 1 h.

The evaluation of the HT pharmacokinetics after its administration through the use of different food matrices evidenced that plasma concentrations were strongly dependent on the matrices used for its consumption (Fig. 2). Hence, with the intake of extra virgin olive oil, the highest average values were found after 30 min (3.79 ng/mL), being the only time-point exhibiting significant differences relative to the control, which showed trace levels below the LOQ ($p < 0.001$). However, HT plasma levels above the quantification limit of the method were found at 30, 60, 120, and 240 min post ingestion, with concentrations ranging between 1.24 and 3.79 ng/mL. A similar trend was observed when comparing the plasma concentration of HT, after the administration of fortified refined olive oil, with the control, which informed on matching times relative to extra virgin olive oil, for achieving the peak concentration of HT, but below the LOQ (traces levels). Again, significant differences with the control were found ($p < 0.01$). The remaining matrices assayed (fortified flax oil, grapeseed oil, margarine, and pineapple juice) did not provide significant increases of the HT plasma concentration HT throughout the 240 min post-intake monitored (Fig. 2).

Urinary excretion of hydroxytyrosol and its metabolites after intake of enriched matrices

As described for plasma samples, the quantification of HT metabolites (HT, HTA, DOPAC, HVAlc, and Tyr) in 24-h urine was performed on hydrolysed samples, which allowed determining the total HT absorbed.

To understand the extent to which the physico-chemical properties of the food matrix influences the bioavailability of HT, results on urine concentration of HT metabolites were statistically processed to determine: (1) the absolute concentration provided by the separate dietary interventions upon the administration of HT in the different matrices (Fig. 3), (2) the increase of HT concentration in urine relative to the basal levels in men and women (Fig. 4a), and (3) the increase of the urine concentration of HT when ingesting it through diverse food matrices (Fig. 4b).

The comparative analysis of the urinary HT after the oral intake of the diverse fortified and non-fortified

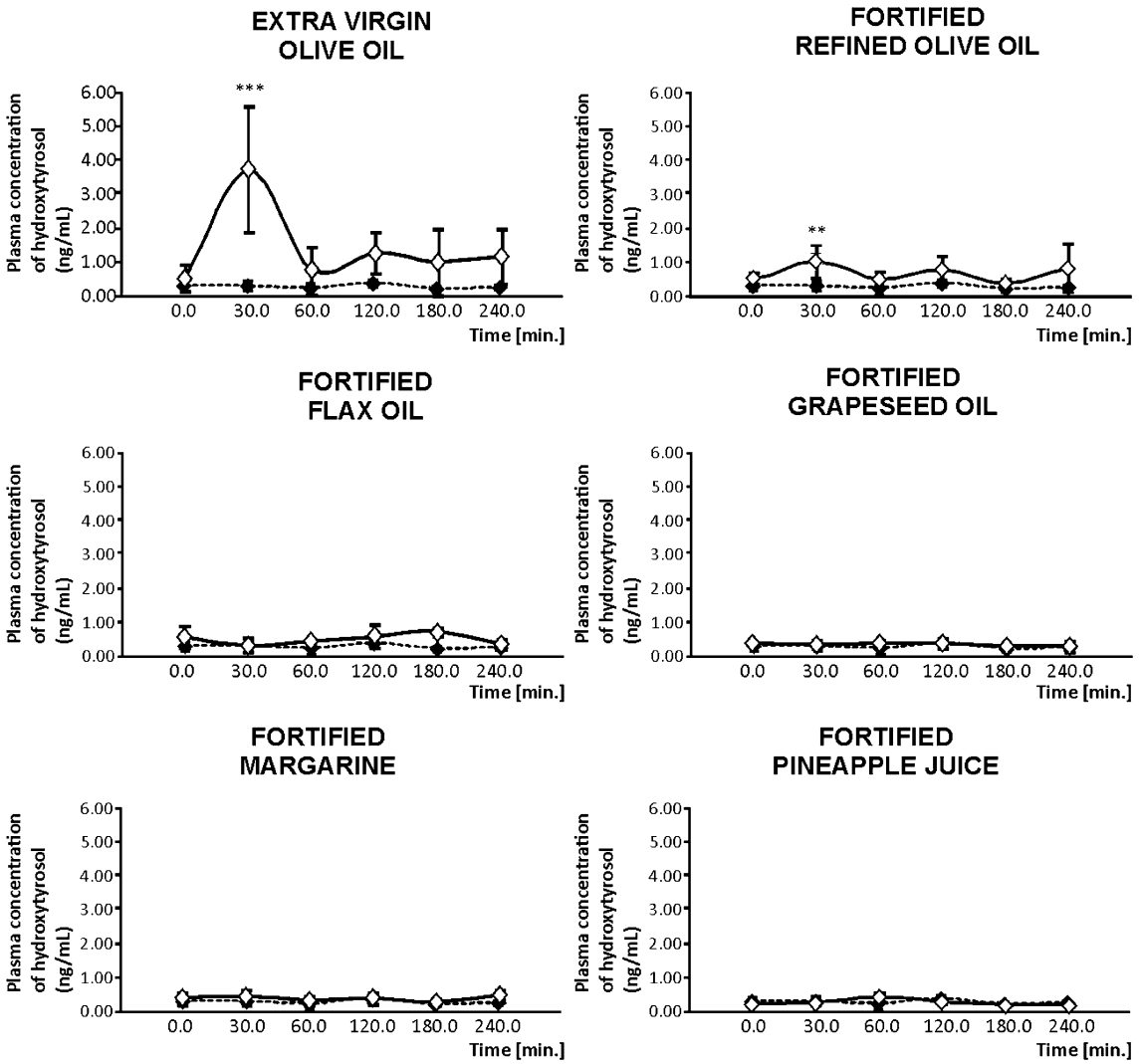

Fig. 2 Pharmacokinetics of HT presented as an overlap of the non-fortified refined olive oil (dotted line) and the specific food matrix at different sampling time-points (0 (Baseline control), 30, 60, 120, 180, and 240 min). Results are expressed as ng/mL. $^{**}p < 0.01$ and $^{***}p < 0.001$

matrices (bioavailability), regardless of the sex of the volunteers, evidenced that the highest concentration was reached when ingesting the 5 mg of HT through the natural source, extra virgin olive oil, which showed a significantly higher urinary content (0.86 µg/mg creatinine in urine at 24 h) with respect to basal urine (Fig. 3). Significant increases of the HT concentration were also found after the ingestion of fortified refined olive oil (0.63 µg/mg creatinine), fortified flax oil (0.55 µg/mg creatinine), and fortified grapeseed oil (0.33 µg/mg creatinine). As for the remaining matrices analysed, refined olive oil (negative control), fortified margarine, and fortified pineapple juice, although the ingestion of HT through these matrices caused an increase in its urine concentrations relative to the basal level, the differences recorded were not statistically significant ($p > 0.05$).

The bioavailability of HT metabolites (HTA, DOPAC, HValc, and Tyr) was analysed by determining their concentrations in urine (reflecting the amount of compound absorbed at the intestinal level or as a result of the metabolism of HT absorbed) (Fig. 3). The results of the urine analysis of volunteers showed no significant modifications of the basal level of the above referred metabolites.

When analysing the increase of the urine concentration of HT, HTA, DOPAC, HValc, and Tyr, considering the sex of the volunteers, it was observed that the highest concentration of HT (34.32 µg/mg creatinine) and HTA (9.58 µg/mg creatinine) in 24-h urine corresponded to men, who surpassed the concentrations recorded in women by 28.2 and 81.4%, respectively, although these differences, because of the inter-individual variations, were not statistically significant (Fig. 4a). With respect to the maximum concentrations

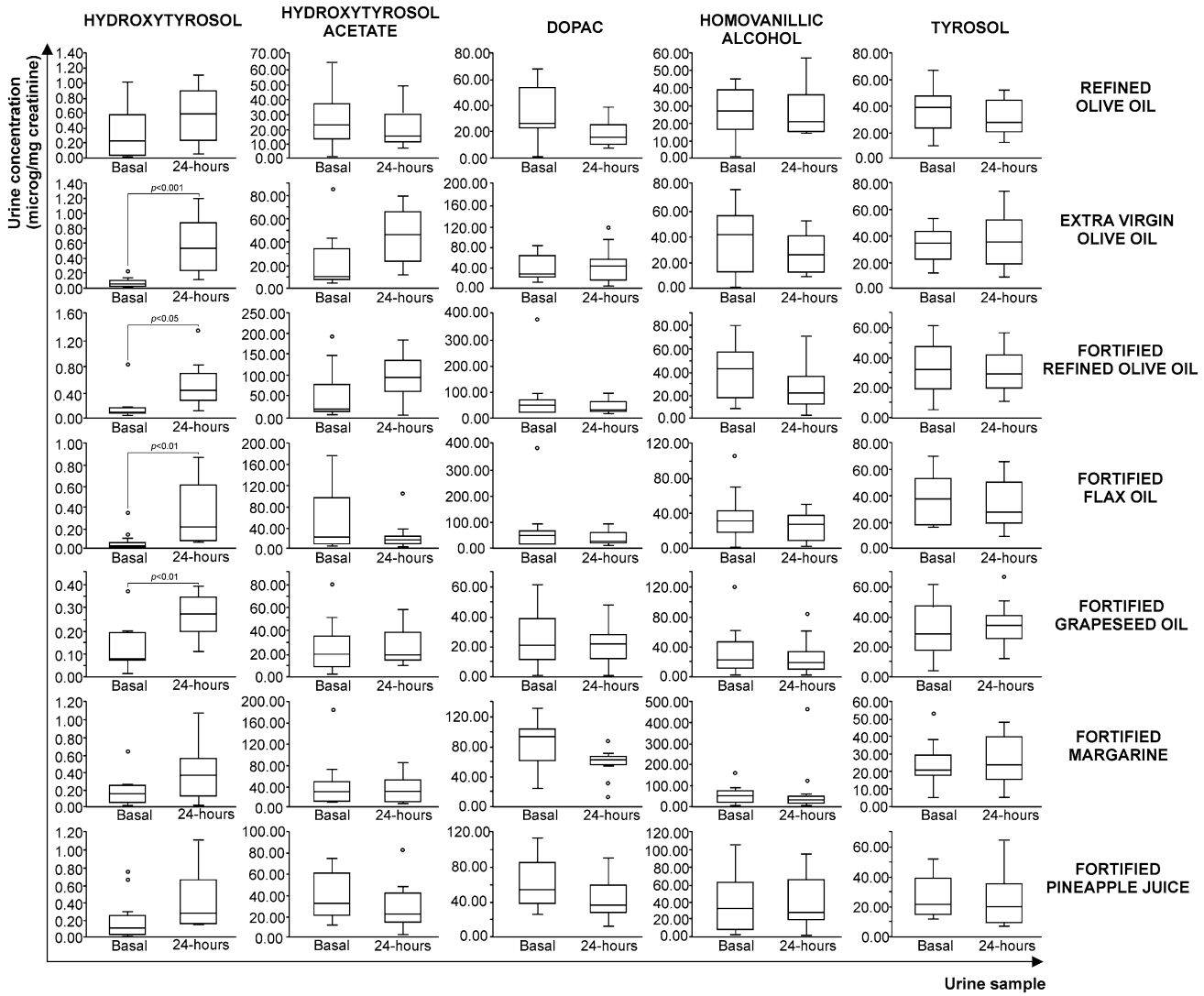

Fig. 3 Bioavailability (μg/mg creatinine) of hydroxytyrosol (HT), hydroxytyrosol acetate (HTA), tyrosol (Tyr), homovanillic alcohol (HValc), and 2,3-dihydroxyphenyl acetaldehyde (DOPAC) was determined in basal and 24-h urine of 20 healthy human volunteers following the administration of 5 mg of HT by diverse matrices: extra virgin olive oil and five fortified food matrices (refined olive oil, flax oil, grapeseed oil, margarine, and pineapple juice). Statistically significant differences between the concentration of the separate HT derivatives in basal and 24-h urine samples were set up at $p < 0.05$, according to unpaired *t*-Student tests

of HValc (3.30 μg/mg creatinine) and Tyr (2.86 μg/mg creatinine), the highest values corresponded to women relative to men (32.2 and 15.9% lower, respectively). As for HT and HTA, the differences in bioavailability of HValc and Tyr between women and men were not statistically significant due to the dispersion of the results. Lastly, DOPAC was found in non-statistically different concentrations in both men and women (4.57 μg/mg creatinine, on average) (Fig. 4a).

When comparing the diverse concentrations of HT in 24-h urine, depending on the intake of the array of fortified and non-fortified food matrices (Fig. 4a), the highest level was obtained when administering HT in extra virgin olive oil (34.20 μg/mg creatinine), which was significantly higher than the rest of the matrices ($p < 0.01$), with the exception of the concentration obtained when ingesting fortified flax oil (10.09 μg/mg creatinine). In this regard, relative to extra virgin olive oil, the ingestion of fortified grapeseed oil and margarine gave rise to concentrations of HT in urine that were 87.5 and 78.9% lower on average, respectively. Moreover, statistically significant lower concentrations of HT in urine were observed when comparing extra virgin olive oil with fortified refined olive oil (9.14 μg/mg creatinine, on average) and fortified pineapple juice (8.95 μg/mg creatinine, on average).

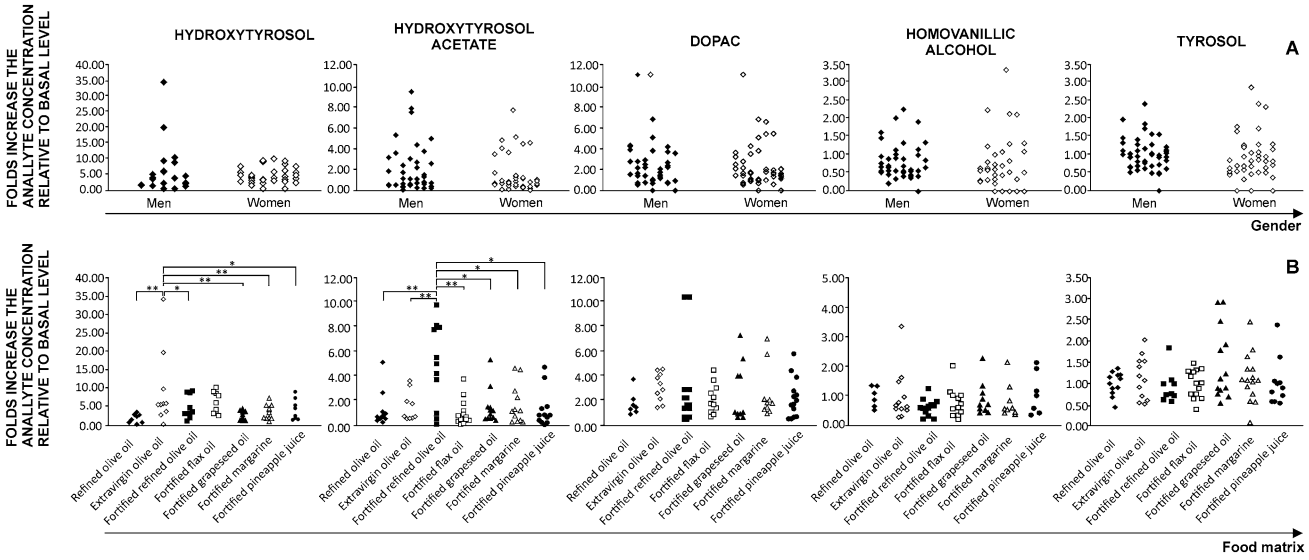

Fig. 4 Increase of the analyte concentration in urine with respect to basal levels according to the sex of volunteers (**a**) and food matrices ingested (**b**)

With respect to metabolites of HT (HTA, DOPAC, HValc, and Tyr) in 24-h urine, after the intake of the different matrices with 5 mg of HT, the only significant difference found corresponded to HTA (Fig. 4b). The highest urine concentration was observed after the intake of fortified refined olive oil (9.58 µg/mg creatinine, on average). When comparing the results of ingesting this matrix with the remaining ones, there were significant differences ($p < 0.01$) with respect to refined olive oil (a 47.9% lower, on average), extra virgin olive oil (a 61.2% lower, on average), and fortified flax oil (a 61.6% lower, on average). As for fortified grapeseed oil, fortified margarine, and fortified pineapple juice, statistically significant lower concentrations were also recorded (5.19, 4.53, and 4.62 µg/mg creatinine, respectively) (Fig. 4b). The analysis of the other metabolites excreted in 24-h urine according to the matrices evaluated, although a trend of increasing levels of DOPAC, HValc, and Tyr was observed after ingesting fortified refined olive oil, extra virgin olive oil, and fortified grapeseed oil, respectively, the differences found were not statistically significant (Fig. 4b).

Discussion

The purpose of this work was to uncover the bioavailability of HT, a phenolic compound with recognized health benefits, especially regarding cardiovascular pathologies, according to scientific opinions published by EFSA and the European Commission [7]. In connection with the recognized interest of HT in the prevention of cardiovascular disorders and its intake through diet, which has given rise to the development of a number of functional foods based on said biological activity, there is a gap of information on the influence of the food matrix on the intestinal absorption and bioavailability of HT and its metabolites. Hence, upon the present work, the differential pharmacokinetic and bioavailability of equal amounts of HT were characterized, after administration through extra virgin olive oil, and an array of food matrices which included refined olive oil, flax oil, grapeseed oil, margarine, and pineapple juice. These matrices feature different physico-chemical characteristics, which were normalized in relation to their concentration of HT to reach the amount recommended by EFSA (5 mg of HT per day) for the achievement of health benefits.

Pharmacokinetics is referred to the study of a given molecule and/or its metabolites' kinetics in the body. It refers to the temporary evolution of a compound and its metabolites in biofluids, tissues, and organs over time [19], while bioavailability informs on the fraction of the ingested nutrient or non-nutrient that is absorbed and available to participate in the various physiological processes or for storage. To the present day, bioavailability has been evaluated by resorting to a plethora of in vitro and in vivo (pre-clinical and clinical) models that provide complementary information [20, 21]. The selection of the model is closely linked to the final objective of the study and strongly conditioned by the availability of previous data on the safety of the compound/s of interest [22]. In addition, it is essential to take into consideration the influence of the diverse in vivo factors such as individual variability, the specific pathophysiological status of volunteers, and the interference of additional components in the food matrix (that could act as competitors or enhancers of intestinal absorption) which may make difficult the in vivo evaluation of bioavailability of bioactive constituents

of foods [23]. Once the safety, biological interest, and bioavailability of bioactive compounds upon in vitro models are demonstrated, the application of in vivo approaches cannot be avoided.

At present, in this regard, information is readily available that supports the close linkage of the bioavailability of phenolic compounds with the physicochemical characteristics of the food matrix upon which they are administered. Thus, in connection with the experimental design implemented in the present work, the administration of HT as an ingredient in various food matrices (extra virgin olive oil, refined oil, pineapple juice, flax oil, margarine, seed oil grape) showed significant differences in pharmacokinetics and bioavailability depending on the matrix considered, which is of high interest for understanding the relevance of selecting the most beneficial diets to obtain the highest benefits of this phenolic compound. Thus, the results retrieved support the high interest in extra virgin olive oil to achieve a dietary source of HT in terms of plasma concentration. However, interestingly, a significant increase of urine concentration of HT was also obtained when ingesting this phenolic HT in diverse matrices (fortified refined olive oil, flax oil, and grapeseed oil). This result is in agreement with previous descriptions, which suggest that oily matrices favour the absorption of HT, although in most cases, the influence of the food matrix was restricted to high *versus* low polyphenolic content in olive oil [24–26], diet supplements either in aqueous solution or in capsules [25] or HT-enriched biscuits (5.25 mg HT/day (30 g biscuits)) [15]. This higher bioavailability has been tentatively attributed to the contribution of additional olive oil components to the absorption of HT [27], and dopamine metabolism [16, 25], although the extent to which the chemical diversity in the food matrix affects the absorption of HT by the intestinal epithelium remains to be elucidated. On the other hand, previous reports have revealed an intense enzymatic activity in the intestinal epithelium which affects HT to a greater extent than its derivatives, thus causing their differential transport through the intestinal lumen to the different organs and tissues [2], a phenomenon that was described early through ^{14}C-isotopic labelling [28].

Previous descriptions available in the literature have also suggested that bioavailability of HT could be conditioned by sex [10, 18, 28]. Taking into consideration this background, and to address the influence of sex in the bioavailability of HT when ingested through the different food matrices, a parity in the distribution of sex of the volunteers was considered in the experimental design of the present study to draw rational conclusions. In this sense, the main absorption events occur in the intestine with the participation of passive diffusion and sodium-glucose co-transporter-1 [29]. Regarding the latter, to the present day, divergent levels in some tissues such as the renal tissue in male and female rats have been described [30]. Moreover, the participation of a diversity of transmembrane proteins (such as breast cancer resistant protein, among others) in the absorption of phenolic compounds has also been observed. This transmembrane protein is also differentially expressed in the liver depending on sex, being responsible for the intestinal absorption of specific chemical forms of xenobiotics. However, the efficiency of the intestinal absorption is not the only factor to be considered when evaluating the bioavailability of phenolic compounds. As stated in previous reports, this could also be influenced by recycling processes found in enterohepatic circulation [10, 31]. Nevertheless, the comparison of the levels of bioavailable HT after oral intake of foods fortified with HT did not show significant differences in relation to the sex of the volunteers, although a higher (non-significant) absorption and bioavailability was observed in men compared with women.

In addition to the study of the levels of HT excreted in urine, for a better understanding of the absorption of HT and, therefore, its bioavailability, it is of great interest to study the metabolites derived from this phenolic compound, despite the remarkable difficulty of identifying these metabolites in biological samples due to their low concentrations and the occurrence in plasma and urine of numerous compounds that could interfere with the ionization of the analytes of interest [17]. Hence, in the present work, this constraint was overcome by the application of liquid–liquid and solid-phase-based clean-up methods that allowed removing unspecific molecules that could interfere with the identification and quantification of the analytes of interest. After using this approach, the results of the assessment of plasma and urine of volunteers after the ingestion of the HT through diverse food matrices, showed that no significant differences were found for HTA, DOPAC, HValc, and Tyr regarding sex. These results, which may seem contradictory relative to other studies characterizing the bioavailability of HT and its metabolites [10, 32], could be attributable to inter-individual variations (not present when analysing bioavailability through pre-clinical models with inbreed animals), although a trend towards increasing and decreasing concentrations of HT acetate and DOPAC, respectively, was observed.

Despite the trend observed regarding the evolution of the concentration of HT metabolites, the interpretation of the results retrieved related to previous works is not a simple task, since no clear pattern was observed between the arrays of matrices evaluated. In this aspect, a study by Domínguez-Perles et al. (2017) [10], after measuring the plasma and urine levels, showed that the increase in the amounts of HT metabolites did not follow linear correlations with its oral administration. The authors of this study concluded that although the diverse metabolites were efficiently absorbed and followed a similar metabolism, their pharmacokinetic and bioavailability are conditioned by a complex network of interactions of the intestinal microbiota with the intestinal

transporters in the first phase of the metabolism of these compounds [10]. Therefore, additional knowledge on such interactions during their phase I metabolism are required by characterizing the occurrence of identified (target metabolomics) and non-identified (untargeted metabolomics) metabolites.

In summary, the results obtained evidence that the absorption and metabolic profile is highly influenced by the food matrix in which HT is incorporated, with the oily nature of the food matrix being especially relevant for the final bioavailability of this phenolic compound, and extra virgin olive oil being highlighted as the best dietary source of this compound. Alternatively, aside from identifying the most relevant source of HT in terms of pharmacokinetics and bioavailability, the results described in the present work would contribute to a better understanding of the relevance of the nature of the food matrices for the final absorption of HT and thus, their potential use as a component for the development of nutraceutical supplements, with a positive impact on the beneficial effects on cardiovascular health, according to the health claims presently recognized for HT.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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